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Report on the Verification of the Performance of MS8, RF3 and GT73 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MS8xRF3xGT73 Oilseed Rape

Validation Report

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Report on the Verification of the Performance of MS8, RF3 and GT73 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MS8xRF3xGT73 Oilseed Rape

21 October 2014

European Union Reference Laboratory for GM Food and Feed

Executive Summary

A joint application was submitted by Bayer CropScience AG and Monsanto Company to request the authorisation of genetically modified stack (GM stack) MS8xRF3xGT73 oilseed rape (tolerant to glufosinate ammonium and glyphosate) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 on GM Food and Feed. The unique identifier assigned to GM stack MS8xRF3xGT73 oilseed rape is ACS-BNØØ5-8xACS-BNØØ3-6xMON-ØØØ73-7.

The GM stack MS8xRF3xGT73 oilseed rape has been obtained by conventional crossing between three genetically modified oilseed rape events: MS8, RF3 and GT73, without any new genetic modification.

The EU-RL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events MS8, RF3 and GT73 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL GMFF has carried out only an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack MS8xRF3xGT73 oilseed rape.

This report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

Content

Quality assurance

The EU-RL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

The EU-RL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by CERMET.

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1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed provides that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (genetically modified stack).

Consequently, the application for authorisation of a GM stack shall be accompanied, among others, by an event-specific method for detection, identification and quantification for each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EU-RL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EU-RL GMFF report in its overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL GMFF carries out an *in-house* verification of the performance of each event-specific method if this method has previously been validated by the EU-RL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack MS8xRF3xGT73 oilseed rape.

Upon reception of methods, samples and related data (step 1), the EU-RL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (EC) No 641/2004 (Annex I).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements [3] and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Bayer CropScience AG and Monsanto Company submitted the detection methods, data demonstrating their adequate performance, and the corresponding control samples DNA extracted from GM stack oilseed rape MS8xRF3xGT73 and from non GM oilseed rape.

The dossier was found to be complete and thus was moved to step 2.

3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL[3] and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated by the applicant for the three methods on the stack DNA. Means are the average of eighteen replicates obtained through three runs performed with ABI Prism® 7900HT Fast sequence detection system. Percentages are expressed in copies as GM DNA / total DNA x 100 (total DNA = GM DNA + non-GM DNA).

Note: Numerical values presented in the tables of this report were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10. The calculations in the MS Excel files however were done over not rounded data. This approach might create generate small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

The EU-RL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria [3].

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) for the MS8, RF3 and GT73 methods applied to GM stack MS8xRF3xGT73.

MS8			
Unknown sample GM%*	**Expected value (GMO %)**		
	0.08	**0.9**	**4.5**
Mean	0.073	0.83	4.8
RSD_r (%)	14	11	6.8
Bias (%)	- 8.5	- 8.2	8.3
RF3			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**4.5**
Mean	0.074	0.89	4.8
RSD_r (%)	11	5.8	7.2
Bias (%)	- 11	- 6.7	6.4
GT73			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**4.5**
Mean	0.074	0.87	4.73
RSD_r (%)	17	5.6	6.2
Bias (%)	- 8.5	- 3.3	5.8

* Unknown samples are DNA samples containing different levels of GM DNA (expressed in copy number) from stack material and non-GM DNA from conventional material.

Two requests for complementary information regarding methods, control samples and DNA sequences were addressed to the applicant. The applicant adapted the previously validated methods introducing small modifications in the reaction volume, primers concentration and in the method of calculation of data (see Section 4.5 for details); the applicant justified these

modifications with the need to improve method's practicability and submitted test results showing that the ENGL method's acceptance criteria were fully satisfied. The EU-RL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

4. Step 3 (EU-RL GMFF experimental testing)

In step 3 the EU-RL GMFF implemented the three methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from leaves of GM stack MS8xRF3xGT73 oilseed rape
- genomic DNA extracted from leaves of non GM oilseed rape

The EU-RL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape and genomic DNA extracted from non GM oilseed rape in a constant amount of total oilseed rape DNA. Table 2 shows the five GM concentrations used in the verification of the MS8, RF3 and GT73 methods when applying them to genomic DNA extracted from the GM stack MS8xRF3xGT73 oilseed rape. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage of MS8, RF3 and GT73 in MS8xRF3xGT73 verification samples.

MS8 GM% (GM DNA / Non-GM DNA x 100)	**RF3 GM%** (GM DNA / Non-GM DNA x 100)	**GT73 GM%** (GM DNA / Non-GM DNA x 100)
0.1	0.1	0.1
0.4	0.4	0.4
0.9	0.9	0.9
1.8	1.8	4.0
3.6	3.6	8.0

The in-house verification followed the protocols already published as validated methods for the individual MS8, RF3 and GT73 events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.2 DNA extraction

A method for DNA extraction from oilseed rape seeds was previously evaluated by the EU-RL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing oilseed rape DNA of appropriate quality and amount for being used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/OSR_DNAExtr_sampl_correctedversion1_CRL_VL_07_04.pdf.

Consequently, the EU-RL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference system *CruA (Cruciferin A).* Five GM levels were examined per run, each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method (MS8, RF3 and GT73), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for the determination of GM%.

4.4 PCR methods

During the verification study, the EU-RL GMFF carried out parallel tests on DNA extracted from GM stack MS8xRF3xGT73 oilseed rape using the single detection methods previously validated for the respective single GM events MS8, RF3 and GT73.

For detection of GM oilseed rape events MS8, RF3 and GT73, DNA fragments of 130-bp, 139-bp and 108-bp respectively are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: FAM (6-carboxyfluorescein) as reporter dye at their 5′-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3′-end.

For quantification of GM oilseed rape events MS8, RF3 and GT73, a taxon-specific reference system amplifies a 101-bp fragment of *CruA (Cruciferin A)* an oilseed rape endogenous gene (GenBank X14555), using two *CruA* gene-specific primers and a *CruA* gene-specific probe labelled with VIC and TAMRA.

For quantification of events MS8 and RF3 DNA in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the amount of MS8 and RF3 events DNA, respectively). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of MS8 and RF3 events, respectively, is estimated.

For quantification of GM soybean event GT73, standard curves are generated both for the GT73, and for the *CruA* specific system by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers and by fitting a linear regression into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves. For relative quantification of event GT73 DNA in a test sample, the GT73 copy number is divided by the copy number of the taxon-specific (*CruA*) reference gene and multiplied by 100 to obtain the percentage value (GM% = GT73/*CruA* x 100).

For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

4.5 Deviations from the validated methods

For MS8 and RF3 events, the methods used by the applicant to generate the data submitted to the EU-RL GMFF in this application were based on the 'two standard curves' approach (one calibration curve for the GM system and one for the reference system). This approach was not substantially affecting the method and produced results well within the ENGL the acceptance criteria. It is however different from the ΔCt approach adopted by the EU-RL GMFF during the validation of the single lines. The applicant asked to the EU-RL GMFF to perform the verification of the MS8 and RF3 methods applied to MS8xRF3xGT73 DNA using the validated methods with no modifications (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

Also for GT73, the method used by the applicant to generate the data submitted to the EU-RL GMFF in the application dossier presented some deviations from the originally validated method (i.e. decrease of reaction volumes from 50uL to 25uL and increase in the final concentration of primers from 150nM to 160nM). These deviations were applied due to availability of different Real Time PCR instruments and did not impact the performance of the method (the results were all within the ENGL limits). The verification study performed by the EU-RL GMFF for GT73 applied to MS8xRF3xGT73 DNA, has been carried out using the method validated for the single line with no modifications (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.6 Results

Tables 3, 4 and 5 present the values of the slopes of the different standard curves generated by the EU-RL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, for GM oilseed rape events MS8, RF3 and GT73.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MS8 method on GM stack MS8xRF3xGT73 oilseed rape.

Run	MS8		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.32	100	1.00
2	-3.38	98	0.99
3	-3.53	92	1.00
4	-3.53	92	1.00
5	-3.30	101	1.00
6	-3.36	98	1.00
7	-3.71	86	1.00
8	-3.38	98	1.00
Mean	-3.44	96	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the RF3 method on GM stack MS8xRF3xGT73 oilseed rape.

Run	RF3		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.45	95	1.00
2	-3.23	104	1.00
3	-3.46	95	1.00
4	-3.58	90	1.00
5	-3.49	94	1.00
6	-3.47	94	1.00
7	-3.51	93	1.00
8	-3.51	93	1.00
Mean	-3.46	95	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the GT73 method on GM stack MS8xRF3xGT73 oilseed rape.

Run	GT73			*CruA*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.46	94	1.00	-3.60	90	1.00
2	-3.53	92	1.00	-3.48	94	1.00
3	-3.56	91	1.00	-3.48	94	0.99
4	-3.40	97	1.00	-3.53	92	1.00
5	-3.61	89	1.00	-3.60	90	0.99
6	-3.49	94	1.00	-3.51	93	1.00
7	-3.35	99	1.00	-3.49	94	1.00
8	-3.51	93	1.00	-3.52	92	1.00
Mean	-3.49	94	1.00	-3.53	92	1.00

The mean PCR efficiencies of the calibration curves for each of the three event-specific methods were above 90% (96% for MS8, 95% for RF3, and 94% and 92% for GT73, respectively). The calibration curves for GT73 and CruA assays were both linear as estimated by the coefficient of determination (R^2), giving a value of 1 for both assays. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the three methods when tested on MS8xRF3xRGT73 in terms of PCR efficiency and linearity.

The EU-RL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) of the three methods applied to samples of DNA extracted from GM stack MS8xRF3xRGT73 oilseed rape, see tables 6, 7 and 8.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MS8 method applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

MS8					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**1.8**	**3.6**
Mean	0.10	0.40	0.96	1.76	3.63
SD	0.02	0.04	0.06	0.15	0.19
RSD_r (%)	18	11	6.3	8.5	5.1
Bias (%)	3.3	1.2	7.2	-2.4	0.8

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the RF3 method applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

RF3					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**1.8**	**3.6**
Mean	0.11	0.43	0.94	1.80	3.83
SD	0.01	0.04	0.08	0.08	0.25
RSD_r (%)	9.3	8.7	8.4	4.5	6.5
Bias (%)	7.4	7.4	4.0	-0.2	6.4

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the GT73 method applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

GT73					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**4.0**	**8.0**
Mean	0.10	0.44	0.97	4.23	8.41
SD	0.02	0.05	0.09	0.25	0.40
RSD_r (%)	16	12	9.0	6.0	4.8
Bias (%)	4.5	9.5	7.9	5.8	5.2

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method should be ± 25% across the entire dynamic range. As shown in Tables 6, 7 and 8, the values range from -2.4% to 7.2% for MS8, from -0.2% to 7.4% for RF3, and from 4.5% to 9.5% for GT73. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

Tables 6, 7 and 8 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the EU-RL GMFF requires RSD_r values to be below 25%. As the values range between 5.1% and 18% for MS8, between 4.5% and 9.3% for RF3, and between 4.8% and 16% for GT73, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

5. Comparison of method performance on MS8xRF3xGT73 and on the single events

An indicative comparison of the performance (bias, RSDr %) of the three methods applied to GM stack MS8xRF3xGT73 oilseed rape and to the single line events is shown in Tables 9, 10 and 11. The performance of the methods on the single lines was previously validated through international collaborative trials (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

Note: the comparison of data generated in different testing conditions and different times is intended to be only of qualitative nature; differences in the figures reported are not necessarily statistically significant.

Table 9. Qualitative comparison of the performance of the MS8 detection method applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape and to genomic DNA extracted from the single line event MS8.

Trueness and repeatability of MS8 quantification on MS8xRF3xGT73			**Trueness and repeatability of MS8 quantification on single event MS8***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	3.3	18	**0.1**	7.4	22
0.4	1.2	11	**0.4**	-3.5	18
0.9	7.2	6.3	**0.9**	-1.0	14
1.8	-2.4	8.5	**1.8**	-1.0	17
3.6	0.8	5.1	**3.6**	-7.5	11

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 10. Qualitative comparison of the performance of the RF3 detection method applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape and to genomic DNA extracted from the single line event RF3.

Trueness and repeatability of RF3 quantification on MS8xRF3xGT73			**Trueness and repeatability of RF3 quantification on single event RF3***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	7.4	9.3	**0.1**	6.9	13
0.4	7.4	8.7	**0.4**	4.4	12
0.9	4.0	8.4	**0.9**	4.5	14
1.8	-0.2	4.5	**1.8**	-2.5	12
3.6	6.4	6.5	**3.6**	-5.2	13

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 11. Qualitative comparison of the performance of the GT73 detection method applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape and to genomic DNA extracted from the single line event GT73.

Trueness and repeatability of GT73 quantification on MS8xRF3xGT73			**Trueness and repeatability of GT73 quantification on single event GT73***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	4.5	16	**0.1**	-25	23
0.4	9.5	12	**0.4**	-13	17
0.9	7.9	9.0	**0.9**	-6.0	17
4.0	5.8	6.0	**4.0**	5.8	14
8.0	5.2	4.8	**8.0**	4.5	14

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

6. Conclusions

The performance of the three event-specific methods for the detection and quantification of oilseed rape events MS8, RF3 and GT73, when applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL.

In conclusion, the verification study confirmed that the three methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack MS8xRF3xGT73 oilseed rape.

Therefore these methods, developed and validated to detect and quantify the single events, can be equally applied for the detection and quantification of the respective events combined in GM stack MS8xRF3xGT73 oilseed rape.

7. References

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unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation (Text with EEA relevance). OJ L 102, 7.4.2004, p. 14–25.

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Abstract

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The EU-RL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events MS8, RF3 and GT73 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL GMFF has carried out only an in-house verification of the performance of each validated method when applied to genomic DNA extracted from GM stack MS8xRF3xGT73 oilseed rape.

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